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Phylogeny of Deltocephalinae（Hemiptera：Cicadellidae）from China based on partial 16S rDNA and 28S rDNA D2 sequences combined with morphological characters

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Abstract: The phylogeny of 19 genera of Deltocephalinae leafhoppers was analyzed based on 50 adult morphological characters combined with nucleotide sequences of the mitochondrial 16S rDNA and nuclear 28S D2 rDNA genes. One species of Typhlocybinae was included as outgroup. Parsimonian，distance and Bayesian methods were used to estimate the phylogenetic relationships. The topology of the phylogenetic trees generated with different methods was quite similar. We partially resolved the morphologically-defined tribes and the relationships among 19 genera of Deltocephalinae. The genus *Macrosteles* was well supported to occupy a basal position in the study，so the most primary tribe in Deltocephalinae might be Macrostelini. The phylogenetic analysis trees put all genera of Deltocephalini but *Nakaharanus* onto a single lineage. The genus *Balclutha*，corresponding to the tribe Balcluthini，remains unsolved in our analyses. The Euscelini might be a polyphyletic group in the analysis. Analytical result recovered Athysanini and Paralimnini as monophyletic clades. The clade *Phlogotettix* and *Scaphoideus-Nakaharanus* was constantly resolved using different methods. We suggested that *Scaphoideus*，*Nakaharanus* and *Phlogotettix* should be included in or into Scaphoideini. But the results resolved poorly the taxonomic status of Xestocephalini overall.

Key words: Hemiptera；Cicadellidae；Deltocephalinae；16S rDNA；28S rDNA；morphology；phylogeny

1 INTRODUCTION

The leafhoppers of Deltocephalinae（Hemiptera：Cicadellidae）are the mainly plant-feeding insects with more than 6 500 species described in the world（Dietrich *et al*.，2001）. Some species are the important pests of agriculture，forestry and industrial crops while some others are well-known as virus transmitters. For example，Nephotettix cincticeps Uhler transmits rice dwarf virus，yellow dwarf virus and rice verticillium. *Hishimonus sellatus* Uhler transmits mulberry dwarf virus. Therefore，deltocephalines are academically and economically important insects.

The Deltocephalinae is one of the largest subfamilies in the family Cicadellidae（Hemiptera）. There existed disputations about the subfamily classification system. Some authors have used the name as Euscelinae（Haupt，1927；Oman，1934；Evans，1947；Emeljanov，1962；Metcalf，1967）while some have adopted the name as Deltocephalinae（Dallas，1870；Ossiannilsson，1983；Anufriev and Emeljanov，1988；Oman *et al*.，1990；Dietrich *et al*.，2001）. Both referred to the same subfamily. Oman（1934）included the Euscelinae 16 tribes，Evans（1947）split the subfamily into 5 tribes and 318 genera，Metcalf（1962，1967）included the subfamily Euscelinae consisting of 2 subfamilies，19 tribes and 390 genera. Oman *et al*.（1990）split the subfamily Deltocephalinae into 22 tribes. The classification system is not resolved yet，and the phylogenetic relationships among Deltocephalinae genera are unclear. The basic taxonomic system relied mainly upon Oman *et al*.（1990）and partly upon Metcalf（1967）in the present paper.

Analyses of molecular data has become a powerful method in the study of phylogeny and has been used in studies of leafhoppers（Fang *et al*.，1993a，1993b，1995；Dietrich *et al*.，1997，1998，2002）. The objective of this study is to examine the phylogenetic relationships among genera of Deltocephalinae using partial 16S rDNA gene and 28S rDNA gene sequences

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combined with morphological data.

The phylogenetic analysis trees based on morphological characters combined with partial 16S rDNA gene and 28S rDNA gene sequences were constructed by maximum parsimony (MP), neighbor joining (NJ) and Bayesian methods in this paper.

2 MATERIALS AND METHODS

2.1 Sampling of taxa

We examined 19 species representing 19 genera in the subfamily Deltocephalinae and one species of subfamily Typhlocybinae as outgroup. The origins of specimens used in this study were listed in Table 1. DNA was extracted from frozen or ethanol-preserved specimens. Fresh specimens were field-collected and frozen in ethanol. For small specimens, DNA was usually obtained from whole body while for large insects DNA was usually obtained from thorax. The remainder of the specimens was saved as a voucher. Vouchers were deposited in Institute of Entomology, Guizhou University, China.

Table 1 Species examined in the study with collecting data and accession numbers in GenBank

Subfamily/Tribe	Species	Collected locality	GenBank accession no.	
			16S rDNA	28S D2 rDNA
Deltocephalinae				
Euscelini	*Hishimonus sellatus*	Guizhou, China	–	AY830688
Euscelini	*Phlogotettix cyclops*	Guizhou, China	AY830913	AY830702
Euscelini	*Goniagnathus punctifer*	Yunnan, China	AY830915	AY830698
Euscelini	*Nephotettix cincticeps*	Guizhou, China	AY830909	AY830701
Euscelini	*Euscelis* sp.	Guizhou, China	AY830907	AY830697
Deltocephalini	*Inazuma dorsalis*	Guizhou, China	AY830916	AY830699
Deltocephalini	*Nakaharanus maculosus*	Yunnan, China	AY830908	AY830690
Deltocephalini	*Paivanana centrifascia*	Guizhou, China	AY830917	AY830692
Deltocephalini	*Doratulina grandis*	Guizhou, China	–	AY830691
Deltocephalini	*Aconura producta*	Guizhou, China	AY830912	AY830695
Deltocephalini	*Deltocephalus bimaculatus*	Guizhou, China	–	AY830696
Macrostelini	*Macrosteles fascifrons*	Yunnan, China	L12998	AY830689
Paralimnini	*Yanocephalus yanonis*	Guizhou, China	AY830911	AY830694
Paralimnini	*Sorhoanus maculipennis*	Guizhou, China	–	AY830700
Athysanini	*Usuironus limbifer*	Guizhou, China	–	AY830693
Athysanini	*Exitianus indicus*	Yunnan, China	–	AY830687
Balcluthini	*Balclutha lineata*	Guizhou, China	AY830914	AY830905
Scaphoideini	*Scaphoideus nigrisiginus*	Guizhou, China	–	AY830903
Xestocephalini	*Xestocephalus japonicus*	Guizhou, China	AY830910	AY830904
Typhlocybinae				
Empoascini	*Empoasca flavescens*	Guizhou, China	–	AY830906

* Sequences from Fang *et al*., 1995.

2.2 DNA isolation

DNA was isolated from individual leafhoppers. The specimen was crushed with a sterile grind stick in a 1.5 mL centrifuge tube, and 300 μL extract buffer (10 mmol/L Tris-Cl, pH 7.4, 10 mmol/L NaCl, 25 mmol/L EDTA) and 30 μL 10% SDS were added. The contents were incubated at 37℃ for 1 – 2 h. Following this step, 30 μL 5 mmol/L NaCl was added and spun in a centrifuge (5 000 rpm) for 5 min. The supernatant was taken into a sterile centrifuge tube and the same volume of phenol-chloroform (25∶24) was added and spun in a centrifuge (3 000 rpm) for 5 min. The homogenate was extracted with phenol∶chloroform∶isoamyl alcohol (25∶24∶1). Then, the supernatant was taken into a sterile centrifuge tube, 1/10 volume of NaAc and 2 volume of 100% ethanol was added and kept 10 – 20 min at room temperature for sediment DNA, then spun in a centrifuge (12 000 rpm) for 10 min. The tube was rinsed 2 times with 70% ethanol. After extraction, the DNA from each specimen was dried and dissolved in 40 μL TE and stored at – 20℃.

2.3 PCR amplification

Two fragments were amplified by PCR, a 550 bp fragment of the 16S rDNA and a 750 bp fragment of the 28S D2 rDNA in D2 region. Reactions were performed in 25 μL total reaction volume (2.5 μL PCR buffer, 1.5 μL MgCls$_2$, 0.5 μL dNTP, 17.8 μL ddH$_2$O, 0.2 μL Taq polymerase, 1 μL template DNA, and 0.5 μL of each primer).

The 16S fragment was amplified using primers 16S-A and 16S-B (Table 2) with the following thermal cycling protocol: 4 min at 94℃; 35 cycles of 1 min at 92℃ (denaturation), 35 s at 47℃ (annealing), 1.5 min at 72℃; and a final extension of 7 min at 72℃

（modified from Fang *et al*.，1993b）.

Amplification of the 28S fragment was accomplished using primers 28S D2-D3（Table 2）and following thermal cycling protocol：3 min at 94℃；20 min at 80℃；30 cycles of 1 min 94℃（denaturation），1 min at 50℃（annealing），2 min at 72℃；and a final extension of 7 min at 72℃（Dietrich *et al*.，2001）.

Table 2　Primers used in PCR amplification and sequencing

Name	Direction	Primer sequence
16S rDNA[a]	Forward	5′-CGCCTGTTTAACAAAAACAT-3′
	Reverse	5′-CCGGTCTGAACTCAGATCAAGT-3′
28S D2-D3 rDNA*	Forward	5′-AGTCGT（K）GTTGCTTGAT（K）AGTGCAG-3′
	Reverse	5′-TTCAATTTCATTT（K）CGCCTT-3′
	Reverse	5′-TTCGGGTCCCAACGTGTACG-3′

[a] These primers were described by Fang *et al*.（1993b）.

* These were essentially the primers described by Dietrich *et al*.（2001）but were modified by replacing“K”with“T”.

PCR products were purified and analyzed by 1% agarose gel electrophoresis stained with ethidium bromide under UV light. PCR products were purified using QIAquick PCR purification kit，resuspended in 20 μL TE（pH 8.0）and stored at －20℃. Both strands were sequenced using ABI 377 automated sequencer with the same primers used for PCR amplification.

2.4　Morphological character analysis

A total of 50 morphological characters（Appendix 1 and Appendix 2）used in leafhopper systematics were selected. Of the 50 characters，13 were from the head，8 from the thorax，8 from the abdomen，and 21 from the male genitalia. Each character was generalized for the genus.

2.5　Sequence alignment

The acquired sequences were deposited in GenBank with accession numbers AY830908 － AY830917 for 16S rDNA，and AY830687 － AY830702，AY830903 － AY830906 for 28S rDNA. The sequences can be downloaded in NEXUS format from web site http://www.Inhs.uiuc.edu/. Sequences were aligned based on distance approach using CLUSTAL X version 1.81（Thompson *et al*.，1997）with different gap opening and gap extension values. Alignments were then adjusted manually based on nucleotides that were obviously misaligned.

2.6　Phylogenetic analysis

Two methods of phylogenetic analysis were performed using PAUP* 4.0（beta 10 version）（Swofford，2001），maximum parsimony（MP）（Farris，1983）and neighbor joining（NJ）（Saitou and Nei，1987）. Bayesian analysis was also used via program MrBayes（3.0）（Huelsenbeck and Ronquist，2001）.

To increase the probability of finding the most parsimonious tree，the heuristic search option was performed on 100 random addition sequences and the TBR option for branch swapping. Each base was treated as an unordered character with equal weight，and gaps were treated as missing. A consensus tree was calculated as more than one most parsimonious tree were found. Consistency of nodes derived with parsimony analysis was treated by bootstrapping with 1000 replications. For obtaining a minimum-evolution tree，a distance-based method following the neighbor-joining algorithm was used. Bootstrapping evaluation of each node was performed with 1 000 replicates.

Bayesian analysis used a simulation technique called Markov chain Monte Carlo（or MCMC）to approximate the posterior probabilities of trees. The command parameters setting of the likelihood model were：lset nst = 2，rates = gamma，ngammacat = 4，ngen = 10 000 000，printfreq = 10 000，nchains = 4.

2.7　Outgroup selection

The Typhlocybinae was relatively close to Deltocephalinae by morphology-based（Hamilton，1983），we used one species of subfamily Typhlocybinae as outgroup in our analysis.

3　RESULTS

Alignment of this dataset resulted in a length of 1 246 characters，containing 1196 nucleotide positions，350 of which were parsimony informative.

We analyzed the combined data to provide a robust estimation of relationships among the 19 genera. Maximum parsimony analysis of this dataset with gaps treated as missing yielded the parsimonian tree which was shown in Fig. 1（Length = 1 783，consistency index = 0.6231，retention index = 0.5327）. A distance-based method following the neighbor-joining was used to obtain a minimum evolution tree（Fig. 2）. The tree yielded from Bayesian analysis was shown in Fig. 3.

The bootstrap values were calculated in parsimonian and distance analyses. Overall，based on the bootstrap，all but two nodes were recovered in more than 50% of the bootstrap replicates in MP tree. In NJ tree 10 nodes had bootstrap support greater than 80%，and all but three nodes were recovered in more than 50% of the bootstrap replicates. All branches supported in the tree yielded from Bayesian analysis were recovered more than 0.5.

The results indicated that the genus *Macrosteles* formed a single lineage，representing a monophyletic group and occupying a basal position in the phylogenetic trees，and this suggests that Macrostelini might be the most primary tribe within the subfamily. However，the genus *Balclutha*，corresponding to the tribe Balcluthini，remains unsolved in our analyses.

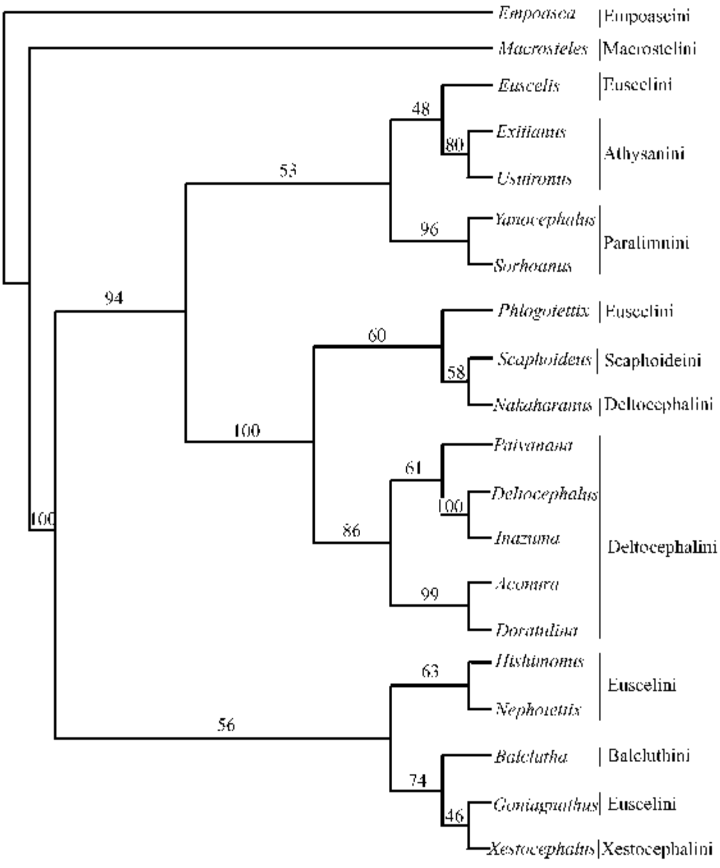

Fig. 1 Phylogenetic analysis using MP methods for morphological data combined with 28S D2 rDNA and 16S rDNA sequences

Numbers above branches are bootstrap values（%）. The same below.

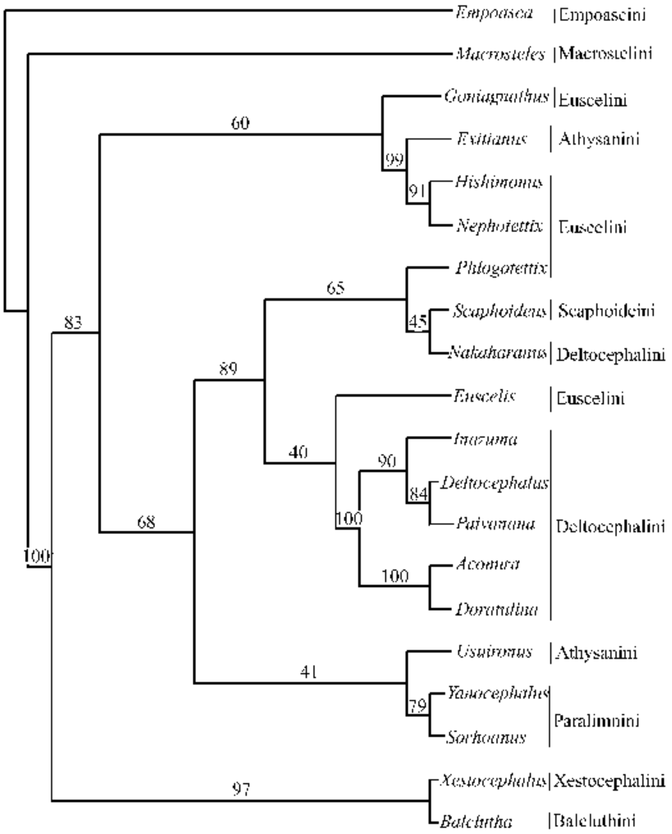

Fig. 2 Phylogenetic analysis using NJ methods for morphological data combined with 28S D2 rDNA and 16S rDNA sequences

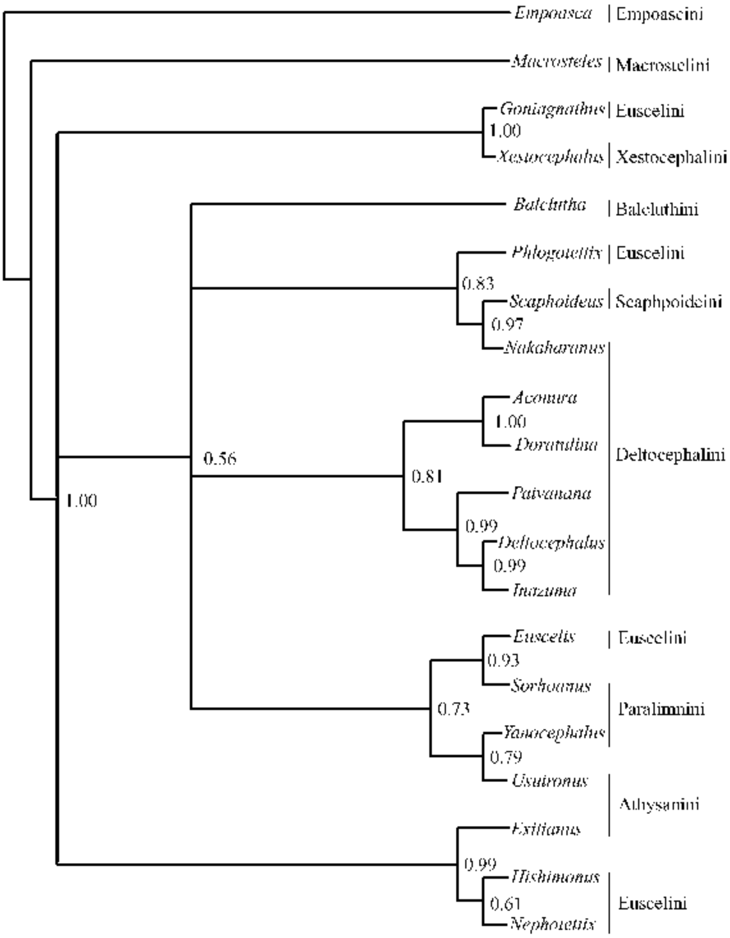

Fig. 3 Phylogenetic tree yielded from Bayesian analyses

The results also indicated that the tribes Paralimnini and Athysanini were the sister group, while Deltocephalini and Scaphoideini formed another clade, same to the suggestions by Metcalf（1962）, Evans（1966）, and Anufriev and Emeljanov（1988）. The Euscelini might be a polyphyletic group in the analyses based on the combining data. The phylogenetic relationships among the genera within the current study indicated that tribe Euscelini needs further study.

The results partially resolved the morphological-defined tribes and relationships among the genera. Nonetheless, the tree resulted from MP method differed from the NJ and Bayesian tree only in including a relationship of *Goniagnathus*（*Xestocephalus* + *Balclutha*）and the placement of *Exitianus* as a sister of *Usuironus*. MP analysis yielded a tree that sustained the position of *Exitianus* as a sister of *Usuironus*, which was the same as the morphological-defined group（Hamilton, 1983）. However, it sustained the relationship *Exitianus* and（*Hishimonus* + *Nephotettix*）in NJ and Bayesian analyses.

4 DISCUSSION

Molecular data has suggested that the large subfamily Deltocephalinae are polyphyletic. However the phylogenetic relationships among tribes and genera remain very pooly understood（Dietrich *et al*. 2001）.

Phylogenetic trees put five of six genera of Deltocephalini but *Nakaharanus* onto a single lineage, showing strong support for a monophyletic Deltocephalini lineage as *Nakaharanus* was removed by morphological characters（vertex round and its vertex length less than pronotal length）. In the tribe Paralimnini, *Yanocephalus* and *Sorhoanus* were clustered on a single lineage in MP and NJ tree. The result strongly recovered the morphologically-defined divisions of the genus（Hamilton, 1983）. But Bayesian analysis yielded the tree that sustained the position of *Yanocephalus* as a sister of *Usuironus*.

In the tribe Deltocephalini, *Aconura* and *Doratulina* were a sister group, which could be defined with morphological characters of vertex anterior margin cone-shaped produced forward in dorsal aspect, and were also consistent with the results analyzed with morphological data (Linnavuori, 1959; Hamilton, 1983).

The clade *Phlogotettix* + (*Scaphoideus* + *Nakaharanus*) was constantly resolved using MP, NJ and Bayesian methods, suggesting its monophyletic nature, which was different from the results from the morphological-defined tribes (Evans, 1947; Metcalf, 1967). The tribe Scaphoideini was created by Oman (1943) for *Scaphoideus* and related genera but was not used at that time. Nast (1972) placed *Scaphoideus* and *Osbornellus* in the tribe Athysanini. Oman *et al*. (1990) recognised the tribe Scaphoideini but there has been no attempt to define valid apomorphies for the tribe. Some authors (*e*. *g*., Metcalf, 1967; Hamilton, 1975; Kamitani, 1999) followed the tribe Scaphoideini. We followed Oman *et al*. (1990) and suggested including *Phlogotettix* + (*Scaphoideus* + *Nakaharanus*) within the tribe Scaphoideini because it was supported by the results both from the analysis based on the 28S D2 rDNA sequences and from the analysis based on the morphological and molecular combined data.

Xestocephalini was considered as a tribe of the Euscelinae (Evans, 1947; Oman, 1949; Linnavuoris, 1959; Metcalf, 1967). Hamilton (1975) proposed Xestocephalina were closely related to the Aphrodina based on their similar genitalia and habit. Subsequently, various authors (Knight, 1974; Hamilton, 1983; Kamitani, 1999; Dietrich *et al*., 2001, 2005) showed that the tribe should be separated as a subfamily Xestocephalinae. The results revealed that there was a close relationship between the genus *Xestocephalus* and *Goniagnathus* in MP and Bayesian analyses, and *Xestocephalus* occupy a basal position. Our results, however, resolved poorly the taxonomic status of Xestocephalini overall.

The combination of morphological and molecular data had a well-resolved phylogenetic estimate for 19 genera of Deltocephaline leafhoppers. However, the position of several taxa remains poorly resolved, as indicated by low bootstrap values and uncertain position of some genera. We would include a larger sample of taxa, the addition of morphological data and sequence data from various regions in future analyses.

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Appendix 1 Morphological characters used in this study and their states and assigned values

1. Ocellus:（0）its diameter or less from eye;（1）remote from eye
2. Interocullar width:（0）greater than vertex length;（1）equal to or less than vertex length
3. Vertex length:（0）less than pronotal length;（1）equal to or greater than pronotal length
4. Vertex:（0）not depressed;（1）depressed
5. Vertex:（0）without fuscous areas or markings, pale or tan;（1）with dedinite fuscous spots or markings;（2）with lateral stripe;（3）mostly or entirely fuscous
6. Vertex:（0）round;（1）slightly produced;（2）strongly produced
7. Head:（0）wider than pronotum;（1）equal to or narrower than pronotum
8. Head, pronotum, and / or scutellum:（0）without strips;（1）with longitudimal strips
9. Clypeal sulcus:（0）prominent;（1）obscure or missing
10. Anteclypeus width:（0）parallel-sided;（1）narrowed apically;（2）wider apically
11. Anteclypeus:（0）1.5 times or less width at midlength;（1）longer
12. Pronotum:（0）lateral margins conspicuous;（1）reduced or inconspicuous;（2）not seen posterior to eye
13. Pronotum:（0）without spots;（1）with spots or longitudinal lines;（2）with lateral line or lines
14. Scutellum:（0）medial length less than pronotum;（1）medial length equal to or longer than pronotum
15. Scutellum:（0）without spots;（1）with conspicuous spots or longitudinal lines;（2）with lateral line or lines
16. Inner anteapical cell:（0）closed basally;（1）open basally;（2）brachypterous not evident
17. Forewings:（0）appendix conspicuous;（1）reduced or absent;（2）submacropterous or brachypterous
18. Forewings:（0）unicolorous or inconspicuous color;（1）with fuscous spots or markings
19. Forewings:（0）without extra crossveins;（1）extra crossveins in clavus;（2）extra crossveins in costa;（3）extra crossveins in both
20. Forewings:（0）macropterous;（1）submacropterous;（2）brachypterous
21. Hindwings:（0）normal（flying）;（1）reduced
22. Pygofer:（0）without process;（1）with process or processes
23. Pygofer:（0）caudal margin broadly rounded;（1）caudal margin truncate or concave;（2）caudal margin conspicuously narrowed apically or acuminate
24. Pygofer:（0）elongate, exceeding anal tube;（1）normal length;（2）truncate or much shortened
25. Plates:（0）triangular or acute apically;（1）rounded apically;（2）truncate apically or otherwise modified
26. Plates:（0）only macrosetae, no hairs;（1）with macrosetae, hairs, or microsetae;（2）with dense tuft or hairs
27. Plates:（0）macrosetae uniseriate, mariginal;（1）macrosetae uniseriate, not marginal;（2）macrosetae not uniseriate
28. Plates:（0）lateral margin linear;（1）lateral margin concave;（2）lateral margin convex
29. Plates:（0）not modified apically;（1）embrowned or otherwise modified
30. Styles:（0）rounded apically;（1）acute;（2）inconspicuous
31. Style with apical lobe:（0）acute;（1）rounded;（2）bifid or otherwise modified
32. Style:（0）apical lobe digitate;（1）apical lobe bifid or otherwise modified
33. Styles:（0）apical lobe without hairs;（1）hairs present on apical lobe
34. Styles:（0）apical lobe not serrate;（1）apical lobe serrate
35. Styles:（0）articulatory arm not produced anterior to lateral arm;（1）articulatory arm produced;（2）articulatory arm not developed
36. Styles with articulatory arm:（0）shorter than style body;（1）as long as or longer than body;（2）not developed
37. Connective:（0）equals or exceeds length of aedeagus;（1）shorter than aedeagus
38. Connective:（0）Y-shaped;（1）linear;（2）U-shaped
39. Aedeagus:（0）articulated with connective;（1）fused with connective
40. Aedeagus:（0）symmetrical;（1）asymmetrical
41. Aedeagus:（0）as long as or longer than style exclude appendages;（1）shorter than style
42. Aedeagus:（0）2 times as wide basally as at midlength of shaft;（1）<2 times as wide basally as at midlength of shaft
43. Aedeagus:（0）not or slightly curved;（1）U-shaped or conspicuously curved
44. Aedeagus:（0）shaft not bifid apically;（1）bifid
45. Aedeagus:（0）shaft not or slightly enlarged apically;（1）shaft enlarged
46. Aedeagus:（0）shaft rounded or ovate at midlength;（1）shaft flattened;（2）shaft troughlike
47. Aedeagus:（0）shaft entire;（1）shaft excavated
48. Aedeagus with gonopore:（0）apical;（1）subapical, dorsal;（2）subapical, ventral
49. Aedeagus:（0）shaft without serrations;（1）shaft with serrations
50. Abdominal segment X:（0）not sclerotized;（1）sclerotized laterally or ventrally;（2）sclerotized dorsally

Appendix 2　Morphological character state matrix

Genera	Character states
Macrosteles	01100100101010000000010000000000000000000001000000 0
Exitianus	11102101100010000000000110010011001010000011000200
Deltocephalus	00100210111000000010000001001100012201101010100010
Hishimonus	00011010001210110100000101220100001010000011000100
Phlogotettix	10010110001201010000012022011101001111010101000201
Nephotettix	10010111001100010120000021011101001000000001100200
Goniagnathus	00003000001200001030012001210221001010010010010202
Usuironus	01001000000001011000002200000101101111200011110202
Paivanana	01101211011110000121001000020121011010010010110101
Aconura	01100211111000001101100100020211002210010010000201
Inazuma	01101210111101010020012012220100000010100000120100
Balclutha	01002001021201010101000100101100000210010110120100
Doratulina	01101100111000022012100001001100012201101010101010
Sorhoanus	01001110020011111110010200120100100010010010000012
Euscelis	00000100001200000000001200000110100010000001110000
Yanocephalus	01101201100020201101100000000100100012010110011201
Nakaharanus	00011010021210101130010000001010000010010011000101
Scaphoideus	00101110011010110120011100110210000010110000000100
Xestocephalus	10000001000110101111000111020100001000000110010100
Empoasca	01100010000000001021010011010100100001001100000100

系统发育分析研究。分析结果表明，二叉叶蝉族 Macrostelini 是一个单系，并在角顶叶蝉亚科的系统发育中处于基部的位置，是内群中最原始的族；角顶叶蝉族 Deltocephalini 中除了纹翅叶蝉属 *Nakaharanus*，其余各属构成单系；殃叶蝉族 Euscelini 内属的归属比较混乱，可能是一个并系群，属间差异有待进一步研究。隆额叶蝉族 Paralimnini 与顶带叶蝉族 Athysanini 是姐妹群。带叶蝉属 *Scaphoideus* 与纹翅叶蝉属 *Nakaharanus* 是姐妹群，二者与木叶蝉属 *Phlogotettix* 的关系最近，三者构成一个单系，建议将三者归为带叶蝉族 Scaphoideini。研究结果还表明，小眼叶蝉族 Xestocephalini 和 Balcluthini 的系统发育位置不明，有待进一步研究。

关键词：半翅目；叶蝉科；角顶叶蝉亚科；16S rDNA；28S D2 rDNA；形态特征；系统发育

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